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An inhibitor of the epidermal growth factor receptor function does not affect the ability of human papillomavirus 11 to form warts in the xenografted immunodeficient mouse model

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Abstract

Epidermal growth factor receptor (EGFr) has been shown to be induced and activated in cells infected with HPV, suggesting that it may play a physiological role in viral replication or in the formation or maintenance of warts. To investigate this possibility, human foreskin tissue was infected with HPV11 and transplanted onto the renal capsule and the dermis of immunodeficient mice. The animals were treated orally or topically with the potent EGFr inhibitor CP-545130, with treatment starting either immediately following graft attachment, or following a 70 day period to allow development of warts. The rate of appearance of warts, wart size and number were monitored. In addition, we measured intra-lesional HPV replication levels and examined the morphology of the graft tissues. Analysis of the results showed no significant difference between placebo and compound-treated groups, despite high levels of compound present in the graft tissue. We conclude that EGFr kinase activity is not required for the development and maintenance of HPV-11-induced warts in this model.

Keywords: HPV; EGFR; Papillomavirus; Epidermal growth factor

1. Introduction

Discovery of new antiviral agents for treatment of genital warts caused by human papillomavirus (HPV) infection has been hampered by the lack of tractable targets. In view of the paucity of viral molecular targets for intervention, several investigators have turned their attention to the interaction with the host cell. HPVs infect the dividing, basal cells of host epithelia and their replicative cycle is intimately tied to the differentiation machinery of these cells. Limited by their small genome size, these viruses have developed multiple strategies to subvert key regulatory circuits that control host cell replication (Howley, 1996). The close association between the cellular proliferation/differentiation state and viral replication provides a potential means to modulate viral proliferation by modifying the growth profile of the infected cells. Among various cellular factors implicated in the regulation of the cellular differentiation program, the EGF receptor (EGFr) tyrosine kinase is a potential target for the development of anti-papilloma agents. Cells infected with HPV over-express EGFr (Johnston et al., 1999; Sizemore and Rorke, 1993), an effect mediated through the viral E5 or E6/E7 proteins (Akerman et al., 2001; Crusius et al., 1997, 1998; Straight et al., 1993; Tomakidi et al., 2000; Wilding et al., 1996). EGFr activation ultimately leads to uncoupling of the processes of cellular differentiation and proliferation, thus allowing HPV replication in cells that would have normally withdrawn from the cell division cycle. Interestingly, EGFr expression and activity are upregulated in many human squamous cell carcinomas (Ozanne et al., 1986), while inhibitors of EGFr induce apoptosis of HPV16-immortalized cells (Ben-Bassat et

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al., 1997) and disruption of the EGFr gene inhibits development of papillomas from HPV16-immortalized keratinocytes (Woodworth et al., 2000).

Taken together, experimental data indicate that EGFr inhibition could result in arrest or reversal of wart growth, and consequently a reversion of the epithelium to a normal phenotype that can no longer support viral replication. To determine whether there is a causal link between EGFr activation and HPV11 replication and wart growth, we tested a well characterized, specific EGFr kinase inhibitor, CP-545130, in an immunodeficient mouse xenograft model, developed and optimized in recent years and increasingly considered to mimic most aspects of HPV replication in the human organism (Howett et al., 1997).

2. Methods

2.1. CP-545130

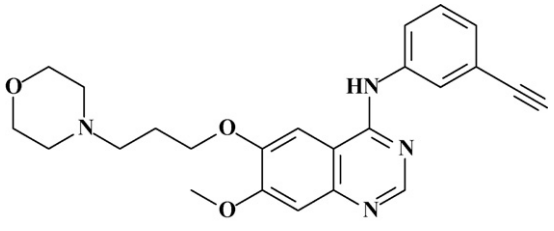
(3-Ethynyl-phenyl)-[7-methoxy-6-(3-morpholin-4-yl-propoxy)-quinazolin-4-yl]-amine, molecular formula $C_{24}H_{26}N_4O_3$ was prepared as described (Arnold and Schnur, 1996). The structure is shown in Fig. 1. A hydrochloride salt was used for all experiments.

2.2. EGFr and selectivity kinase assays in vitro

Inhibition of the phosphorylation of poly glutamic acid: tyrosine (PGT, Sigma Chemical Co., St. Louis, MO) by EGFr or other recombinant kinases was measured in vitro using an ELISA method as described previously (Moyer et al., 1997). Preparation of recombinant EGFr, IGFr and v-Abl was performed as described by Moyer et al. (1997). Baculovirus-expressed cytoplasmic domain of the insulin receptor β subunit (10 units/well) was purchased from Stratagene, and purified human platelet c-src (1.2 units/well) was obtained from Oncogene Science Inc. The intracellular domain of ErbB2 (residues 675–1255) was expressed as a GST fusion protein in baculovirus-infected Sf9 cells. Recombinant protein was purified by affinity chromatography on glutathione sepharose beads. Screening for selectivity against a panel of kinases was performed as previously described (Davies et al., 2000).

2.3. Tyrosine phosphorylation in cells

Cells from the EGFr-overexpressing breast cancer cell line MDA-MB-468 were seeded in 96-well plates in Dulbecco's Modified Eagle's Medium and incubated for 24 h at 37 °C, 5% CO_2 in a humidified atmosphere. Compound dissolved in DMSO was added to the cells over a range of concentrations. Cells were incubated for a further 16 h, then the amount of EGFr activity was determined as described by Pollack et al. (1999).



CP-545130

Fig. 1. The molecular structure of CP-545130.

2.4. Compound formulation for in vivo experiments

Formulation and administration of CP-545130 to HN5-grafted mice was carried out as previously described (Pollack et al., 1999). For the treatment of HPV11-induced wart grafted mice, CP-545130 was formulated for oral delivery in 10% (v/v) DMSO (Sigma)/0.1% (v/v) Lutrol F127 (BASF) vehicle. The doses used in the experiment were selected based on in vivo inhibition of EGFr-associated tyrosine phosphorylation in human tumor tissue xenografts by CP-545130 (Pollack et al., 1999). For topical delivery, solubility was measured in a range of generally recognized as safe (GRAS) excipients which are precedented for topical applications. Chemicals were from Sigma unless otherwise stated. Compound was dissolved in the different excipients in duplicate by shaking overnight at room temperature. The samples were centrifuged to remove any insoluble compound prior to analysis by HPLC to determine solubility.

2.5. Transdermal flux

Transdermal flux was determined using a Microette Transdermal Diffusion Cell autosampling system with static Franz diffusion cells and 4.5 ml receptor compartments. A saturated 2 ml solution of CP-545130 was prepared in a glass vial and the pH was adjusted to pH 5.0. The vial was rotated on a blood tube rotator for 48 h at room temperature, then centrifuged at 15,000 rpm for 60 min. The supernatant was removed and used to determine transdermal flux [^{14}C] mannitol (3 μCi/ml) was included as a control to check the membrane integrity. The reservoirs of the diffusion cell apparatus were filled with PBS at pH 7.4 which was stirred with a magnetic stirrer at 300 rpm and maintained at 37 ± 1 °C resulting in a skin surface temperature of 32 ± 1 °C, mimicking in vivo skin surface temperature. Human cadaver skin at a thickness of 300–500 μm was stored at −20 °C. Approximately 18 h prior to the experiment, 20 mm × 20 mm squares of skin were thawed at room temperature and hydrated in 0.002% (w/v) sodium azide in water. The skin was sandwiched between the donor and receptor compartments of the apparatus with the stratum corneum side facing the donor compartment. The apparatus was allowed to equilibrate for 1 h prior to application of 200 μl of CP-545130 and [^{14}C] mannitol solution to the exposed surface of the epidermis (0.63 cm^2). Six diffusion cells were used. Receptor fluid of 1 ml from each cell was collected into vials automatically at pre-programmed intervals over a 24 h period. The removed receptor fluid samples were replaced with 1 ml of fresh PBS from reservoirs. Mannitol flux was determined by scintillation counting of the collected aliquots. Membranes were assumed to be intact if mannitol flux did not exceed 3% after 18 h. At the end of the experiment, the stratum corneum was removed from the skin samples by adhesive tape stripping method and the remaining tissue was dissolved in a solution

of 1 M sodium hydroxide: Triton X-100:methanol:water (3:1:3:1) at 60 °C for 48 h. Acetonitrile treatment was used to precipitate protein from the samples and following centrifugation, CP-545130 concentrations were determined by HPLC-MS in comparison to a range of standards prepared by spiking known concentrations of CP-545130 into fresh sections of skin.

2.6. Tumor growth inhibition and EGFr phosphotyrosine determinations in vivo

All animal procedures were carried out in compliance with federal guidelines and institutional policies. The tumor growth inhibitory effects of CP-545130 were measured in 3–4-week old athymic female mice (CD-1, *nu/*nu) bearing established, palpable (2–4 mm diameter) human HN5 tumors, as described by Pollack et al. (1999). Eight animals were used in each treatment group. Compound was dosed orally once a day in a 10% DMSO/0.85% sodium chloride/0.1% Pluronic P105 (v/w/w) vehicle. Tumor data collected from the final day measurements were evaluated for statistical significance as described below. Compound-induced inhibition of EGFr-associated tyrosine phosphorylation in human tumor explants from athymic mice was determined using the ELISA assay described by Pollack et al. (1999). Four animals were used in each treatment group. As the tumor volume and EGFr tyrosine phosphorylation measurements provide data on a continuous scale, the one way analysis of variance (ANOVA) was used for statistical analysis of the relationships between groups. The significance level was assigned at 0.05 and p values were determined using Dunnett's t statistic. A set of internal laboratory standards (aliquots from previously frozen tissue for both treated and control groups) was used to assess the quality and reproducibility of the immunoassay.

2.7. Animal models for HPV11-induced wart growth

Female athymic mice (*nu/nu* on BALB/c background) and female beige/nude/scid mice (*bg/nu/xid*) were purchased from Taconic Farms, New York. Both were housed in flexible film isolators and supplied with sterile air, water and autoclaved laboratory chow supplemented with vitamins. Human foreskin tissue was obtained from routine neonatal circumcision at a local hospital. Split thickness skin chips (1 mm × 1 mm × 0.5 mm and 10 mm × 10 mm × 0.5 mm) were cut with a scalpel and incubated either with HPV11 Hershey extracted from previous experimental condylomata, or with control saline for 1 h at 37 °C. The small size chips were inserted beneath the renal capsules of nude mice as described previously (Kreider et al., 1985, 1986). Multiple samples from multiple donors were used and these were randomly distributed across different treatment groups. Large size chips were transplanted to an orthotopic site on the backs of *bg/nu/xid* mice (Howett et al., 1997). Each mouse received a chip from a different donor. The experiment commenced after a period of 2 weeks to allow graft attachment and vascularization. A minimum of 10 mice were used for each test group.

CP-545130 was administered orally once a day to animals with renal capsule grafts at 1, 10 and 100 mg/kg. Two different treatment protocols were used. In the first, treatment was commenced immediately following the 2-week graft attachment and vascularization period and continued for 56 days. The second protocol was designed to mimic the clinical situation where patients present with established infection, thus treatment was delayed until day 70 when the grafts were transformed, then continued for 30 days. For topical treatment, 25 mg of 0.2% or 2% (w/v) CP-545130 was applied once a day to the orthotopic grafts with treatment commencing immediately following the 2-week graft attachment and vascularization period and continuing for 56 days. In both oral and topical treatment arms, 3 control groups were included. These were uninfected grafts treated with placebo (compound vehicle only), uninfected grafts treated with 100 mg/kg CP-545130 (oral) or 2.0% CP-545130 (topical) and HPV-infected grafts treated with placebo.

For renal capsule grafts, the animals were euthanased at the end of the experiment and the kidneys removed, fixed in 10% neutral buffered formalin and embedded in paraffin. Serial sections were mounted onto silane-coated microscope slides and examined histologically for the presence of HPV DNA by in situ hybridization (Unger et al., 1991). In cases where the grafts had been unsuccessful, no further analysis was carried out. For successful grafts, they were scored qualitatively as positive or negative for transformation and the presence of HPV DNA by in situ hybridization with HPV11-specific riboprobes (Stoler et al., 1990). Orthotopic grafts were examined daily and the time of papilloma appearance and the number of grafts which developed papillomas, was noted. At the end of the experiment, the animals were euthanased; the grafts were washed with PBS then removed. Due to the small size of the tissue, grafts for each treatment group were pooled into two batches which were independently assayed for the presence of compound as described above for transdermal flux experiments.

As the data from the HPV experiments are categorical, rather than continuous, the Pearson's Chi-squared test was used for statistical comparison between the groups.

3. Results

3.1. CP-545130 selectively inhibits EGFr tyrosine kinase in vitro

CP-545130 (Fig. 1) is one of a series of inhibitors of EGFr phosphorylation, known to possess potent activity against the enzyme in vitro and in tissue culture, as well as in vivo animal models (Moyer et al., 1997; Pollack et al., 1999). We found CP-545130 to be a potent inhibitor of EGFr kinase-mediated phosphorylation of PGT in vitro, with an IC_{50} of 2.5 nM (Fig. 2A). The compound also inhibited EGF-induced phosphorylation of EGFr in MDA-MB-468 cells with an IC_{50} of 15 nM (Fig. 2B). To ensure that the observed activity was specific and to confirm selectivity, we measured the potency of CP-545130 against a range of tyrosine kinases. IC_{50} determinations for the compound showed >200-fold selectivity over erbB2 and >1000-fold selectivity over 4 other tyrosine kinases

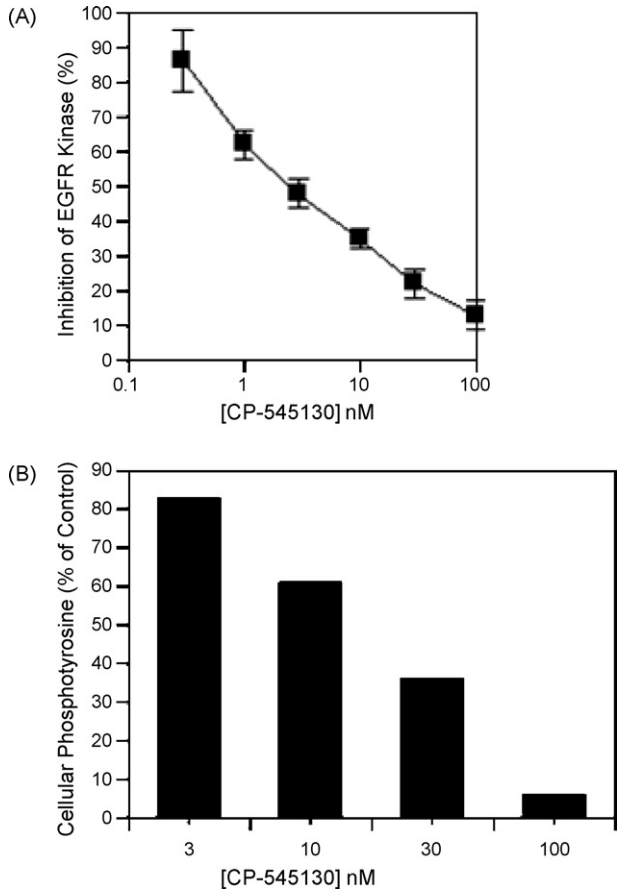


Fig. 2. (A) CP-545130 inhibition of EGFr-mediated phosphorylation of PGT in vitro. Figures are mean ± S.E. (B) CP-545130 inhibition of EGFr activation in MDA-MB-468 cells treated with inhibitor for 24 h and activated with 50 ng/ml EGF. Figures are mean of duplicate samples, which agreed within 10%.

(Table 1, left). Further in vitro screening at a single concentration of 10 μM showed that CP-545130 had no activity against a panel of 29 kinases, with the exception of Lck (77% inhibition; Table 1, right).

3.2. Treatment of mice with CP-545130 inhibits EGFr phosphorylation and growth of grafted tumor cells

HN5, a head-and-neck squamous carcinoma cell line, is known to over-express EGFr and has been shown to be

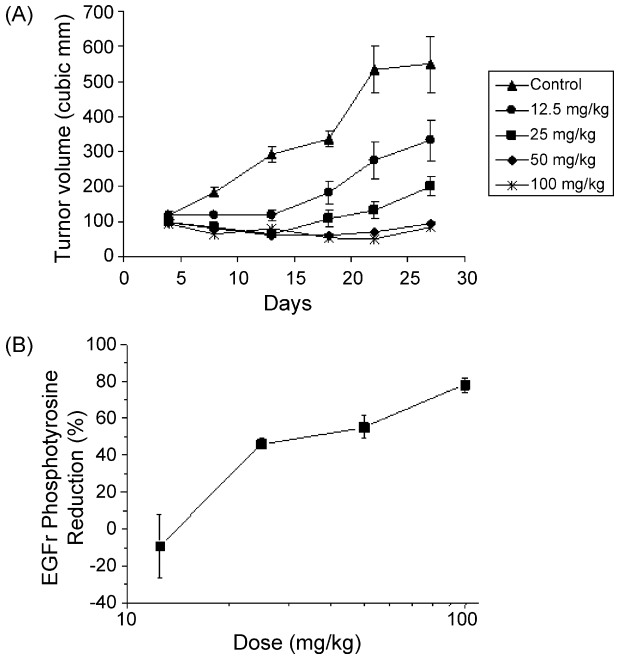


Fig. 3. Inhibition of HN5 tumor cell growth and EGFr activity in subcutaneously grafted mice treated with CP-545130. (A) HN5 tumor size following oral administration of CP-545130. Mean tumor volumes ± S.E. are shown. (B) Inhibition of EGFr activity in grafted HN5 cells 1 h following oral dosing of CP-545130. The mean reductions of tumor phospho-EGFr ± S.E. are shown.

dependent on EGFr function for growth both in vitro and in vivo (Pollack et al., 1999). To investigate whether the activity of CP-545130 against the receptor translates to an anti-tumor effect in the context of a whole animal, we administered the compound to HN5 grafted mice and measured both the effect on the tumor cell growth and on EGFr autophosphorylation. CP-545130 produced significant dose-related antitumor effects against established HN5 growing subcutaneously in athymic mice (Fig. 3A). When test animals were dosed for 20 consecutive days, beginning at 4 days after tumor implantation, the ED_{50} was 10 mg/kg/day. The minimum effective dose for significant antitumor effect was 6.25 mg/kg/day (data not shown; $p < 0.05$ with Dunnett's test) and the effects with doses of 12.5 mg/kg/day and higher (Fig. 3) were also all statistically significant ($p < 0.05$). Tumor stasis was achieved during the dosing period for all doses equal to, or greater than, 25 mg/kg/day. Consistent with this anti-tumor effect, we also showed that CP-545130 inhibited

Table 1
Activity of CP-545130 against a panel of 6 tyrosine kinases expressed as IC_{50} (left) and against a panel of 29 additional protein kinases expressed as % inhibition at 10 μM (right)

Kinase	IC_{50} (μM)	Kinase	% Inhibition at 10 μM	Kinase	% Inhibition at 10 μM	Kinase	% Inhibition at 10 μM	Kinase	% Inhibition at 10 μM
EGFr	0.0017	MKK1	<10	PKCa	<10	SAPK2a	36	PP2a	26
Abl	>10	ERK2	30	PDK1	<10	SAPK2b	<10	CK2	3
Src	>10	JNK	<10	PKBa	<10	SAPK3	<10	Lck	77
ErbB2	0.35	MSK1	<10	SGK	27	SAPK4	<10	CSK	<10
IGFR	>10	PRAK	<10	ROCK-II	11	MAPKAP-K1a	11	CDK2	<10
InsR	>10	PKA	<10	AMPK	13	MAPKAP-K2	<10	CK1	11
		CHK1	15	DYRK1a	15	P70S6K	<10	GSK3b	<10
		Phosphorylase kinase	26						

phosphorylation of EGFr in the grafted HN5 cells when measured one hour after administration, with an ED_{50} of 35 mg/kg (Fig. 3B).

3.3. Oral treatment of mice with HPV-infected renal capsule grafts

Having demonstrated that CP-545130 inhibits EGFr phosphorylation and EGFr-dependent tumor growth in vivo, we investigated the efficacy of CP-545130 against HPV11-transformed human foreskin samples grafted under the renal capsules of athymic mice. The compound was dosed orally at 1, 10 and 100 mg/kg/day in two different treatment regimes. The 100 mg/kg/day dose is well in excess of concentrations previously shown to cause tumour stasis and inhibition of EGFr phosphorylation in grafted human cells. In the first treatment regime (protocol A), compound dosing was commenced immediately following the 2-week graft attachment and vascularization period and continued for 56 days (Fig. 4A). The second protocol (protocol B) was designed to mimic the clinical situation where patients present with established infection, thus treatment was delayed until day 70 when the grafts were transformed, then continued for 30 days (Fig. 4B).

The success rate for grafting of the foreskin samples was variable and was not affected by either treatment with CP-545130 or infection by HPV11 (data not shown). In all treatment groups, a minimum of 15 successful grrafts was obtained. Morphologic examination of the surgically removed grafts indicated that as expected, transformation was only observed in samples that had been infected with HPV11 prior to being implanted. The rate of transformation of infected samples was similarly high in both placebo- and compound-treated animals (mean 42.6% in the protocol A group and 48.2% in the protocol B group), with no values significantly different from the controls (Figs. 4 and 5A). Similarly, there was no difference in the amount of HPV DNA

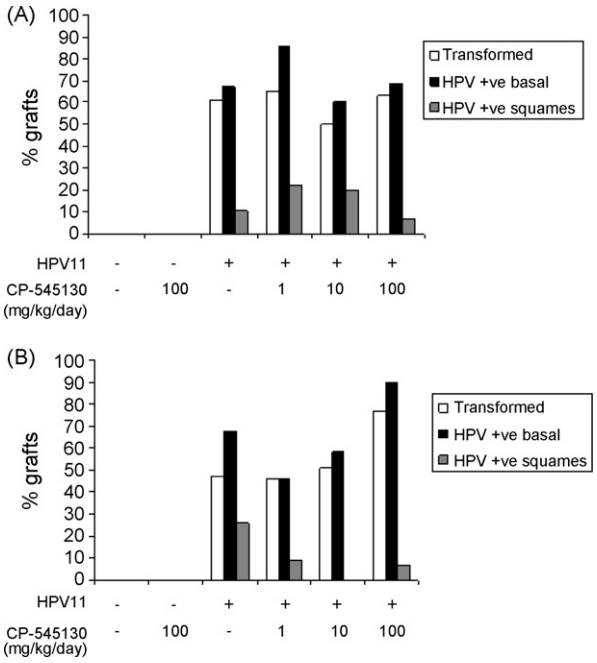


Fig. 4. Effect of orally administered CP-545130 in a mouse HPV11-infected renal capsule xenograft model. (A) Treatment was commenced immediately following the 2-week graft attachment period and continued for 56 days. (B) Grafts were left for 70 days to allow transformation to occur, and then treatment was carried out for 30 days. Grafts were examined for transformation and in situ hybridization was carried out to detect HPV DNA in the basal layer (latent infection) or squames (productive infection). Figures are number of grafts expressed as a total of successful grafts.

detected by in situ hybridization of the foreskin samples between placebo-treated and compound-treated animals (Fig. 5B), or the number of grafts positive for HPV DNA (Fig. 4). Interestingly, HPV DNA was more frequently present in the squames of placebo-treated animals from protocol B than in the squames of treated animals (31% of placebo-treated versus <11% for any

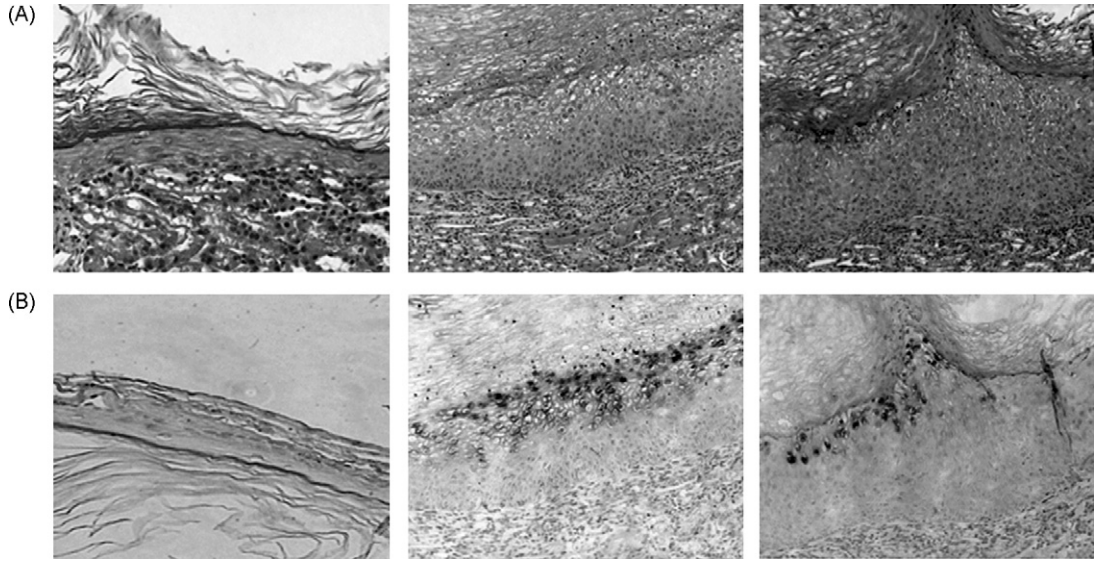


Fig. 5. Histology and in situ hybridization of HPV11-infected human foreskin implanted under the renal capsule of athymic mice. (A) Histology slide of tissue from uninfected graft (left), HPV-infected and treated with placebo (middle), HPV11-infected and treated with high dose of CP-545130 (right). (B) In situ hybridization of the above sections with HPV11-specific DNA probe.

of the three treatment groups, p-value <0.01). Surprisingly, such difference is not apparent in samples from protocol A, where treatment is initiated prior to establishment of the productive infection and is longer in duration. This could indicate that the above observation may be due to the variability inherent to the methodology. Alternatively, there may be a time-dependence of the effect of EGFr inhibitors on HPV replication (see Section 4). In conclusion, no dose-dependent inhibition of either graft establishment, transformation or HPV infection was seen in either of the treatment regimes.

3.4. Topical treatment of HPV-infected orthotopic grafts

The orthotopic graft system mimics the clinical disease associated with HPV11 more closely than the renal capsule graft model and could therefore be more relevant for the evaluation of experimental inhibitors. In addition, topical treatment of the lesions enables application of higher concentrations of compound directly to the tissue. In order to identify a suitable formulation for those experiments, the solubility of CP-545130 was examined in a range of excipients which have previously been used for topical delivery. A solution of 30% glycerol/60% ethanol/10% water (v/v/v) showed good solubility over a range of pHs (>15 mg/ml) and was chosen for further studies. To investigate the ability of this formulation to deliver CP-545130 to skin, trans-epidermal delivery (TED) experiments were carried out. A saturated solution of compound in 30% glycerol/60% ethanol/10% water (v/v/v) at formulation pHs ranging from 2.3 to 7.8 was applied to the stratum corneum of human cadaver skin and the amount retained in the skin was determined. All formulations achieved high skin concentrations (data not shown). The formulation at pH 4.9, which had a solubility of 17.9 mg/ml and achieved a skin concentration of 2.1 mM, was chosen for testing in the animal model. In addition, we determined that the compound is chemically stable across the pH range 2.0–9.0 in aqueous conditions at accelerated temperature conditions of 121 °C. Two formulations of CP-545130 (0.2 and 2%, w/v in 60% ethanol/30% glycerol/10% H_20 (v/v/v) at pH 4.5–5.0) were used for topical delivery to HPV11-foreskin grafted mice.

Treatment was commenced immediately following the 2-week graft attachment and vascularization period and continued for 56 days. Throughout the experiment the development of papillomas on the grafted tissue was monitored (Fig. 6). At the end of the experiment, treated grafts were removed, washed, and the tissue concentration of CP-545130 was determined by HPLC-MS (Table 2). As with the orally treated mice, CP-545130 caused a small, but not statistically significant, reduction in the proportion of grafts that transformed (36% in the low dose and 33% in the high dose, compared with 46% in the placebo-treated group; p-value 0.79). Compound treatment did not significantly affect the lag period prior to the appearance of papillomas, despite the presence of high concentrations of the EGFr inhibitor. These observations are consistent with the outcome of the oral administration studies, and indicate that EGFr inhibition does not impact HPV11-induced transformation of orthotopically grafted human foreskin samples.

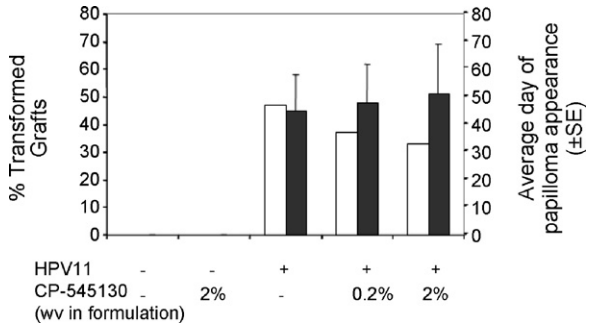


Fig. 6. Effect of topically administered CP-545130 in a mouse HPV11-infected orthotopic xenograft model. Percentage of successful grafts which showed transformation is shown in white bars. Average day of papilloma appearance is shown in black bars.

Table 2
Concentration of CP-545130 in treated orthotopic grafts

Treatment	Concentration of CP-545130[a] (μg/g of graft tissue)
Uninfected + placebo	<10
Uninfected + 2% CP-545130	269 ± 11
Infected + placebo	<10
Infected + 0.2% CP-545130	132 ± 9
Infected + 2% CP-545130	275 ± 117

[a] Grafts from each group were divided into two samples and compound concentration was determined by HPLC-MS. Figures are mean ± S.E. Limit of quantification of the assay was 5 μg/g.

4. Discussion

We have explored the proposed link between EGFr activation and HPV replication using a potent inhibitor of EGFr function. Using a well-characterized mouse xenograft model, we investigated the effects of this compound on wart formation induced by HPV11. In the initial experiment, CP-545130 was administered orally to mice with HPV11-infected human foreskin tissue grafted beneath the renal capsule, at concentrations up to 100 mg/kg/day over a period of up to 56 days. This study failed to identify any significant effect of treatment with CP-545130 on either the maintenance of HPV DNA in the grafts, or on the morphology of the infected tissue. Although the small amount of graft tissue did not allow us to demonstrate inhibition of EGFr in this experiment, it is worth noting that CP-545130 was found to both inhibit EGFr auto-phosphorylation (ED_{50} = 35 mg/kg) and retard EGFr-dependent human tumor cell-line growth (ED_{50} = 10 mg/kg/day) at doses well below the highest doses used in our experiments. The lack of significant effect in the HPV system indicates that EGFr activity is not required for establishment and maintenance of a HPV11 infection and transformation in this model.

To ensure that the above outcome is not biased by the site of grafting, we confirmed our findings in mice with orthotopic human tissue grafts. In this system, human foreskin tissue is grafted onto the dermis of *bg/nu/xid* mice, thus placing the human cells in their natural context, with regard to temperature and exposure to the atmosphere among other parameters. In addition, we treated the grafts early (post-establishment

and vascularization, but prior to appearance of warts) with a purpose-developed topical formulation capable of delivering compound at high concentrations. At the end of the experiment, we measured a concentration of 275 μg/g CP-545130 in the graft tissue of the high dose group. As with the previous experiment, the small size of graft tissue did not allow us to demonstrate inhibition of EGFr in the tissue, however the concentration of compound detected in the grafts at the end of the experiment was several orders of magnitude greater than the observed in vitro potency of the compound (EGFr IC_{50} 15 nM, equivalent to 6.3 ng/ml) and would therefore be expected to have completely inhibited EGFr function. Despite such high levels of compound being present in graft tissue and prolonged treatment periods (56 days), we did not see any significant inhibition of wart formation. It should be noted that in the in vitro TED studies, the stratum corneum was stripped off before determining the concentration of compound in the skin sample, demonstrating that the compound can readily penetrate the lower levels of skin. Given the potency of the inhibitor, the high concentrations applied and the prolonged duration of dosing, we believe that concentrations well above the IC_{50} in vitro and the EC_{50} in vivo have been achieved in the skin. From our experiments, we conclude that in vivo, EGFr activity is not required for the establishment of a productive HPV11 infection and resulting tissue transformation.

Numerous studies demonstrate a correlation between HPV infection and EGFr up-regulation or activation. However there is little evidence to suggest that EGFr activity is a requirement rather than a consequence of the infection or transformation. Furthermore, most studies linking HPV and EGFr have been carried out using the high-risk types HPV16 or HPV18. There are reports that the low risk HPV6 E5 protein can associate with EGFr (Conrad et al., 1994), that EGFr activity is increased in laryngeal papillomas (caused by HPV6 or HPV11) and that these undergo morphological changes when their growth medium is depleted of EGF (Johnston et al., 1999; Vambutas et al., 1993). However, there is no published data explicitly linking HPV11 infection with EGFr activation. It is therefore possible that HPV11 does not interact with EGFr nor require its activity for replication, and that different host growth factors would be required for establishment and proliferation of distinct HPV subtypes. This hypothesis is worth further consideration, as should it be true, EGFr inhibitors may still be useful in inhibiting replication of other low-risk or high-risk HPV subtypes.

A further significant difference between our work and previously reported studies resides in the methodology that we followed. Much of the previous work indicating that EGFr activity modulates HPV replication has been carried out using transformed primary cells and cell-lines, or ex vivo grown tissue. It is conceivable that in vitro conditions alter the physiology of the tissue or transformed cells in a way that renders them more stringently dependent on EGFr activation. It is also possible that tissue growing in mice is exposed to a number of growth factors and other regulatory stimuli absent in vitro, which compensate for the effect of EGFr inhibition. Indeed, numerous studies indicate that there could be a link between HPV replication and and other growth factor receptors (Lopez-Ocejo et al., 2000; Walker et al., 2003; Venuti et al., 2002; Conrad et al., 1994). It is therefore possible that there are multiple pathways by which HPV can stimulate cell proliferation. Inhibition of only one of these may not be sufficient to prevent wart formation. This hypothesis could also explain the observed reduction in HPV DNA-positive squames following a 30-day, but not a 56-day treatment. It is conceivable that when treating the grafts for longer time periods, a homeostatic event (possibly taking advantage of alternative growth factor pathways) re-establishes permissiveness of the tissue to HPV.

Finally, while we believe our model to be a good mimic of the clinical manifestations of HPV11 replication, it is limited by the lack of a fully functional immune system and human soluble factors. It is conceivable that EGFr activation would be a compensatory mechanism to a human inhibitor of wart formation or viral growth, or that it plays an active part in immune evasion. In either case, reliable demonstration would require ad-hoc clinical studies.

In summary, our data show that in the models used, EGFr is not required for establishment and maintenance of a productive HPV11 infection and formation of lesions. Additional studies are required to ascertain whether this finding can be extrapolated to clinical HPV11 infections in humans. Whether these conclusions should be restricted to this particular HPV subtype, or that the frequently described over-expression or activation of EGFr is a consequence rather than a requirement for HPV infection merits further investigation.

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